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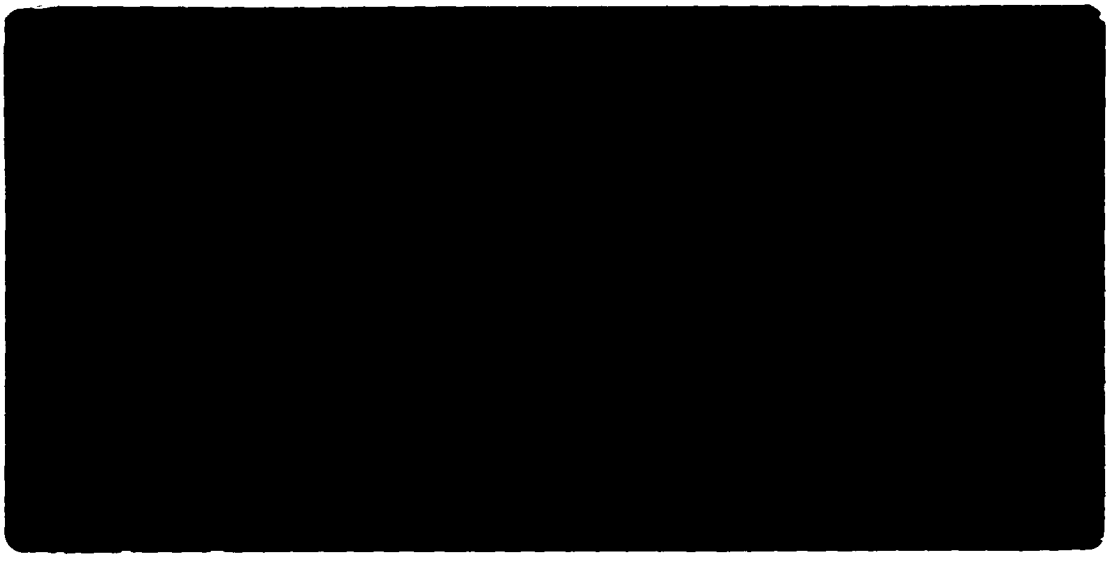

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UTILIZING CAENORHABDITIS ELEGANS TO BIOMONITOR TOXICITY OF METALS IN MILL EFFLUENTS

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Teri A. Ard and Ronald J. Dinus

ABSTRACT

Current methods used by the pulp and paper industry for determining toxicity of effluents have been found to be expensive, time-consuming, and of uncertain reliability. A nematode, Caenorhabditis elegans, has been investigated as a bioassay to evaluate toxicity of mill effluents. Numerous compounds are found in mill effluents, including heavy and transition metals which are being given increasing attention. C. elegans was used to test the toxicity of Al, As, Cd, Cr, and Ni. Experimental results indicated high precision and reproducibility within laboratories as well as between laboratories. The use of C. elegans for toxicity testing is expected to yield more highly reproducible results that those obtained with other test organisms such as Ceriodaphnia or fathead minnow at less expense.

INTRODUCTION

With increasing public attention on the environment, the pulp and paper industry is concerned with numerous compounds found in mill effluents. Several methods are available for determining the chronic toxicity of industrial effluents. Ceriodaphnia and fathead minnows are commonly used by the pulp and paper industry for internal monitoring of effluents and also by the EPA for regulatory inspections. Both methods are expensive, time-consuming, and of questionable reliability.[1,2] A nematode, C. elegans, has been used to develop and evaluate a quick, inexpensive, and

reproducible toxicity test method. Intra- and interlaboratory results are indicative of the high reproducibility of this test method.

BACKGROUND

A relatively unknown but potentially useful procedure for chronic toxicity measurements utilizes a nematode, Caenorhabditis elegans. This nematode is an approximately one mm long "worm" and is the most thoroughly characterized animal known.[3] It is the only animal whose entire cell lineage has been determined from a fertilized egg to all 810 cells in the somatic tissues of the adult.[4] The connectivity of all 302 neurons has also been described.[5]

C. elegans has been used as a biological model in numerous areas of research including genetic mutations, aging studies, neurological evaluations, and medical applications. Researchers acknowledge that convenience and ease of study are the major benefits of utilizing this nematode in test methods.[5,6] Specifically, these organisms are hermaphrodites, or self-fertilizing; therefore, populations are genetically uniform. Also, a population can be maintained indefinitely without genetic drift by nitrogen freezing the stock.

The advantages associated with the use of nematodes for toxicity testing are relevant to pulp and paper industry needs. Along with effluent testing, methods are also needed for sediment and sludge toxicity testing. Because C. elegans is a free living soil species, this organism has the capability to be adapted for this purpose. Therefore, one test organism could be used to monitor the potential toxicity of effluents, sludges, and sediments.

Benefits inherent to the nematode method also include large sample sizes (300-1000), simplified feeding and living conditions, and a short three-day life cycle. Other key attributes include its translucent anatomy to facilitate observation and its suitability for genetic analysis. The low cost of maintaining and testing this species is also an important attribute.

Even with a perfect procedure, the question arises, "how does this test method relate to human chronic toxicity"? C. elegans and humans have similar neuromuscular junctions and transmitters such as acetylcholine, serotonin, norepinephrine, gamma amino butyric acid (GABA), and dopamine.[7] The nematode is obviously not as complex an organism as a human, but its simplicity heightens reproducibility. Nematode determined LC_{50} values have also been found to parallel LD_{50} values ascertained by other animal testing methods.[3] Nematode methods are capable of determining LC_{50} values which correlate with toxicity effects of marine species.[8] With these capabilities, nematodes could provide a vital link between land animals, marine wildlife, and humans.

Unfamiliarity with nematode methods is the largest disadvantage. These methods have not been listed or reported by the EPA. Neither the EPA nor any other environmental regulatory agency has developed or proposed a protocol.

EXPERIMENTAL MATERIALS AND METHODS

Materials. Caenorhabditis elegans var. Bristol (strain N2) was graciously provided by Dr. David Dusenbery, Georgia Institute of Technology. C. elegans can be stored and maintained for definite periods of time by isolating the nematodes in a larval, or dauer, stage which is specialized for long-term survival. During this stage, dauer larvae do not

feed and are resistant to stress. Large numbers of dauer larvae were obtained by utilizing an egg white procedure.[9] The dauer larvae were then maintained in a M9 buffer solution in erlenmeyer flasks at 20°C with no exposure to light.[5] Twenty-four hours prior to beginning a toxicity test, an aliquot of solution containing dauer larvae was placed in a 100mm petri dish containing 30 ml of nematode growth medium (NGM)[5] and a lawn of Escherichia coli strain OP50 as a food source. E. coli was cultured and maintained in a 37°C incubator.

After washing and centrifugation, an E. coli pellet was resuspended in test solution. Three ml of test solution was pipetted into a 60mm Pyrex petri plate. These plates were then placed in the 20°C incubator for 24 hours to equilibrate conditions. Ten nematodes from a 24-hour matured dauer stock, now young adults, were then transferred via a 32 gauge platinum wire to each plate.

Experimental Design. A minimum of five concentrations of each metal ion were tested for toxicity, along with a control. Each concentration and control was replicated with 10 plates and 10 nematodes per plate (100 individuals). Each experiment was designed to statistically eliminate time and operator effects.

Test metal ions included Nickel(Ni), Aluminum(Al), Chromium(Cr), Arsenic(As), and Cadmium(Cd). Metals were administered to the C. elegans via the following reagent-grade metallic salts: $CdCl_2$, $NaAsO_2$, $K_2Cr_2O_7$, $NiCl_2$, and $Al(NO_3)_3 \cdot H_2O$.

Plates containing test solutions were removed from a 20°C incubator every 24 hours to evaluate nematode survival. Survival determinations were done using a transmitted light microscope, since reflected light can lead to dehydration and

subsequent mortality of the specimens. Death was defined as a total lack of response to prodding with a wire. Worms not found were assumed dead and decomposed beyond recognition.

Data Analyses. Toxicity was quantified every 24 hours by expressing the number of surviving nematodes as a percentage of the original number. This percentage represents survival rate and was evaluated further with statistical analyses.

LC_{50} is defined as the concentration of metal ion where the mortality level of the test population is equal to 50 percent. Each LC_{50} was determined by linear regression of survival data based on the log of the metal ion concentration.

RESULTS AND DISCUSSION

In our view, an important quality in a test method is the reliability of the test method not only to determine toxicity, but also to produced consistent and reproducible results between laboratories as well as within laboratories. By choosing compounds that had previously been evaluated by C. elegans, determinations of both inter- and intralaboratory reproducibility could be ascertained. The only compounds that have been evaluated for aquatic toxicity by C. elegans were metal compounds reported by Williams and Dusenbery of the Georgia Institute of Technology.[8,10] Since metals are present in mill effluents and sludges and are potential sources of toxicity, they were used as our test compounds.

Interlaboratory Reproducibility. Toxicity results are reported in Table 1. These LC_{50} values were compared to Williams' values. Arsenic and chromium results, expressed in terms of the log of LC_{50} in ug/l, are illustrated in Figures 1 and 2. Since

only a limited range of concentrations were tested, results did not warrant specifing the LC_{50} in terms as specific as ug/l; therefore, the log of the LC_{50} is used. It can be seen that LC_{50} values determined for arsenic directly correspond to the results reported earlier by Williams. Another metal, chromium, produced results that correlate well for days one, two, and three. However, as can be seen in Figure 2, there is a discrepancy between determined LC_{50} values for day four. This is a trend that was repeated with aluminum, cadmium, and nickel as well.

Table 1. LC_{50} values for metals determined by Ard and Dinus.

LC_{50} (mg/l)	Day 1	Day 2	Day 3	Day 4
Aluminum	34	12	6	2
Arsenic	195	181	175	176
Cadmium	295	246	93	43
Chromium	229	18	7	11
Nickel	363	200	30	6

FIGURE 1. LC_{50} VALUES FOR ARSENIC.

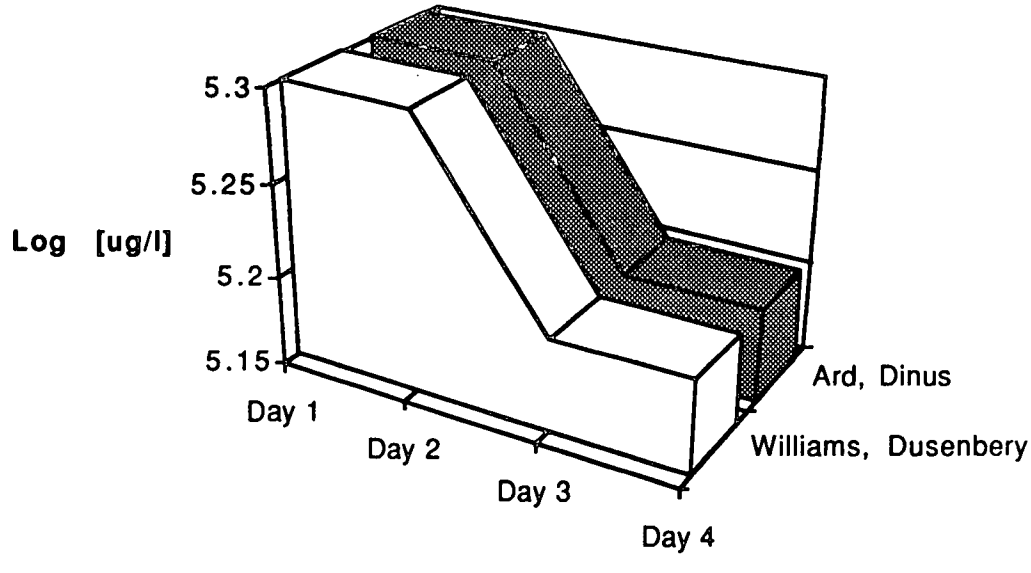
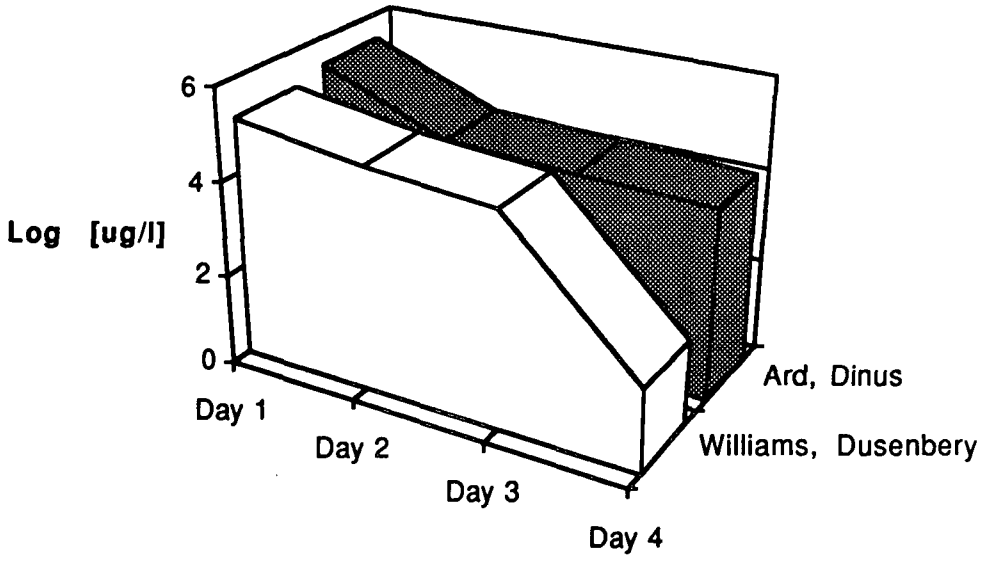


FIGURE 2. LC_{50} VALUES FOR CHROMIUM.



After further evaluation, it is believed that the discrepancies resulted as a consequence of variations in laboratory feeding procedures. This hypothesis is currently being investigated by evaluating effects of various levels of E. coli upon nematode survival and reproduction. By optimizing the reproduction of the population, the optimal nutritional needs can be determined. It appears that in this study, the nematodes were fed a larger dose of E. coli than the nematodes in Williams' study. This is due to a lack of procedural quantification of nutritional needs. No protocol or method has been proposed to quantify the amount of E. coli fed to the C. elegans. If the nematodes are better fed, in the longer term, they would be healthier and potentially less susceptible to toxicity effects of metals resulting in higher survival rates.

Intralaboratory Reproducibility. Since the experimental plan was set up in a randomized block design, metal results were analyzed using standard analysis of variance techniques to determine significant variation caused by repetitions and

treatments. Each metal and day were analyzed separately. Results indicate that there were significant effects among treatments, but there were no significant differences among repetitions.

In the case of nickel, aluminum, and cadmium, the experiment was separated into two sets of five repetitions each. Each subdivision was done on separate weeks so as to determine potential time effects. No differences between subdivisions were discovered. As examples, Figures 3 and 4 specify the distribution of survival results for aluminum(day 1, acute toxicity) and nickel(day 4, chronic toxicity). Each set of the experiment, assayed on separate weeks, is denoted uniquely in the figure. Some points are not visible due to overlapping data points.

FIGURE 3. ALUMINUM DAY 1 SURVIVAL DISTRIBUTION.

10 data points per concentration

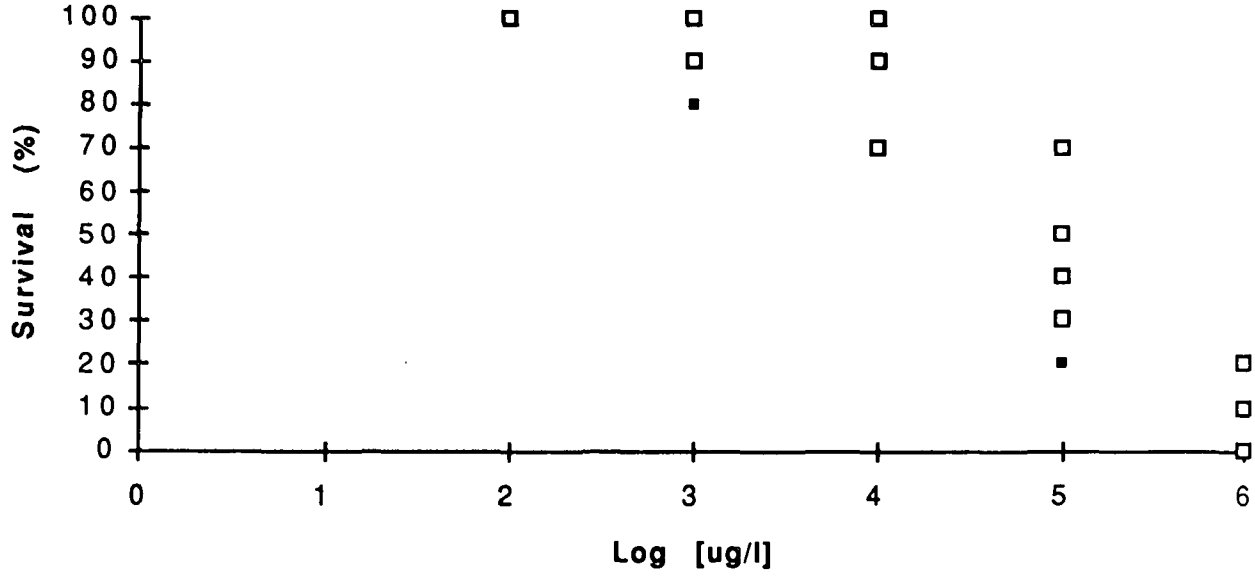
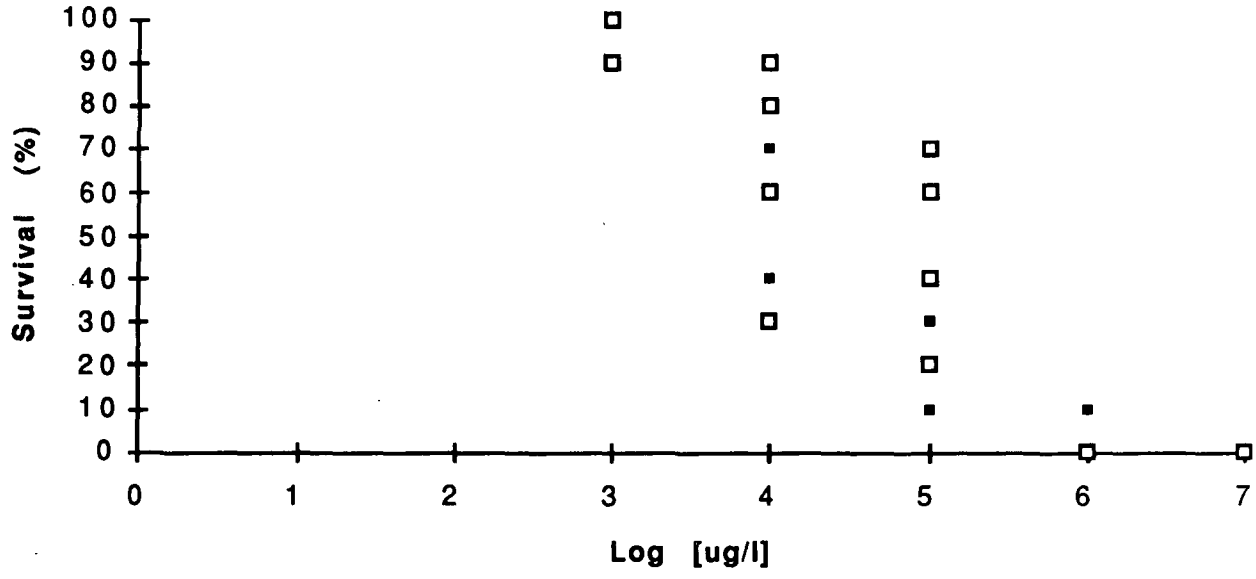


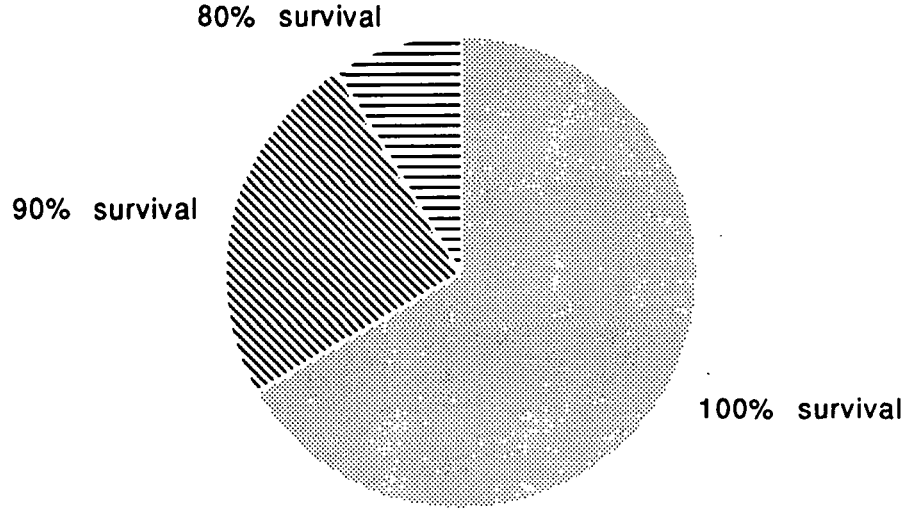
FIGURE 4. NICKEL DAY 4 SURVIVAL DISTRIBUTION.

10 data points per concentration



Control survival is another important issue in toxicity test methods. For example, inconsistent Ceriodaphnia survival in control solutions has been cited as a major issue with this method.[1,2] During our study, a total of 50 control dishes, with 10 nematodes per dish, were maintained and scored. The average day 1 survival for controls was 100%. On the last day of the toxicity test, day 4, the average survival for control dishes was 96%. Figure 5 illustrates the distribution of survival for individual control dishes. Thirty-three of the 50 control dishes have 100% survival for the four days of the mortality tests, and no plate had less that 80% survival.

FIGURE 5. SURVIVAL DISTRIBUTION FOR INDIVIDUAL CONTROL DISHES FOR DAY FOUR.



Finally, time and financial considerations influence the utility of a toxicity test method. Labor requirements for Ceriodaphnia and fathead minnow chronic tests are high. Approximately 90 hours of labor are needed over a 7-day test period. Culture maintenance and expenses are additional.[2] On the other hand, our experience in the lab has shown that a 4-day test period requires less that 35 hours of labor.

CONCLUSIONS

Utilizing the nematode Caenorhabditis elegans as a method to evaluate the acute and chronic toxicity of mill effluents is a viable alternative that necessitates further investigation. Based on the research presented here, C. elegans provides information on toxicity of metals that is reproducible both between laboratories and within laboratories.

Additional work needs to be done to standardize and quantify the amount of E. coli fed to the C. elegans during the test period. Furthermore, with the establishment of reproducibility of laboratory results, emphasis can be placed on evaluating compounds to which C. elegans has not previously been exposed and for which toxicities may not be known. Use of this user-friendly test method will permit identification of troublesome compounds, assessment of the individual hazards, and will provide a more thorough analysis of current and proposed process technology.

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